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Comparison of *In Vitro* and *In Vivo* Estrogenic Activity of UV Filters in Fish

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In this work, we evaluate whether *in vitro* systems are good predictors for *in vivo* estrogenic activity in fish. We focus on UV filters being used in sunscreens and in UV stabilization of materials. First, we determined the estrogenic activity of 23 UV filters and one UV filter metabolite employing a recombinant yeast carrying the estrogen receptor of rainbow trout (rtERα) and made comparisons with yeast carrying the human hERα for receptor specificity. Benzophenone-1 (BP1), benzophenone-2 (BP2), 4,4-dihydroxybenzophenone, 4-hydroxybenzophenone, 2,4,4-trihydroxy-benzophenone, and phenylsalicylate showed full dose–response curves with maximal responses of 81–115%, whereas 3-benzylidene camphor (3BC), octylsalicylate, benzylsalicylate, benzophenone-3, and benzophenone-4 displayed lower maximal responses of 15–74%. Whereas the activity of 17β-estradiol was lower in the rtERα than the hERα assay, the activities of UV filters were similar or relatively higher in rtERα, indicating different relative binding activities of both ER. Subsequently, we analyzed whether the *in vitro* estrogenicity of eight UV filters is also displayed *in vivo* in fathead minnows by the induction potential of vitellogenin after 14 days of aqueous exposure. Of the three active compounds *in vivo*, 3BC induced vitellogenin at lower concentrations (435 μg/l) than BP1 (4919 μg/l) and BP2 (8783 μg/l). The study shows, for the first time, estrogenic activities of UV filters in fish both *in vitro* and *in vivo*. Thus we propose that receptor-based assays should be used for *in vitro* screening prior to *in vivo* testing, leading to environmental risk assessments based on combined, complementary, and appropriate species-related assays for hormonal activity.

Key Words: **UV filters; *Pimephales promelas*; vitellogenin; *in vitro-in vivo* comparison; fish estrogen receptor α; human estrogen receptor α.**

INTRODUCTION

Numerous studies have focused on compounds that are agonists for estrogen receptors α and β (ERα, ERβ) (Routledge and Sumpter, 1997; Schultz *et al.*, 2000; Sohoni and Sumpter, 1998; Soto *et al.*, 1991). These include a wide range of different compounds having different molecular structures and binding affinities that may induce effects in wildlife (Jobling *et al.*, 1998; Vos *et al.*, 2000). With regard to the considerable number of chemicals that are and will be assessed for possible estrogenic activity, *in vitro* systems play an important role for identification and first screening of estrogenic compounds that interact with the ER. Within the framework on the Endocrine Disrupters Testing and Assessment (EDTA) of the OECD, animal and non-animal tests are proposed (OECD, 2002). For assessing possible ecotoxicological effects, a fish *in vivo* screening assay is planned, but no *in vitro* assays using fish-based systems have been proposed (OECD, 2004). Considering the vast number of compounds to be tested for aquatic systems, it is important to employ appropriate *in vitro* systems for fish (Ackermann *et al.*, 2002; Le Guével and Pakdel, 2001; Pakdel *et al.*, 2000). In this regard, the question arises as to what extent *in vitro* systems can mimic *in vivo* activity of estrogenic compounds. *In vitro* systems are cost-effective tools and allow for rapid screening of a large number of compounds, but they have limitations, which may result in unreliable predictions. Therefore, the most appropriate way to determine the endocrine-disrupting activity of chemicals seems to include both *in vitro* and *in vivo* assays, as no single assay may be best suited to determine the hormonal activity of a compound. In our present work, we address this question, focusing on important chemicals in personal-care products found in the aquatic environment.

Sunscreens and cosmetics including lipsticks, skin lotions, hair sprays, hair dyes, shampoos, and numerous other products contain increasing amounts of compounds protecting from ultraviolet (UV) radiation. Either organic UV filters, or inorganic micropigments (ZnO, TiO_2) scattering and reflecting UV light, or combinations of both, are applied. Increased

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sunlight-protection factors are being used for preventing negative effects on the human skin, which generally requires higher percentages of UV filters in the products. Combinations of different UV filters are increasingly employed for absorbing UVA, UVB, and UVC light.

Inputs of UV filters into the aquatic system occur directly via recreational activities (bathing) into surface water, and indirectly via wastewater. Ultraviolet filters are photostable, many of them highly lipophilic (log Kow 3–7) and relatively stable in the aquatic environment (Balmer *et al.*, 2005; Poiger *et al.*, 2004), which makes these compounds critical for bioaccumulation. Residues of several UV filters have been detected in human milk (Hany and Nagel 1995) and in fish (Balmer *et al.*, 2005; Nagtegaal *et al.*, 1997), in the latter between 21–3100 ng/g lipid, and also in lakes and wastewater, with maximum concentrations up to 125 ng/l (Poiger *et al.*, 2004) and 19 μg/l (Balmer *et al.*, 2005), respectively.

At present, the estrogenicity of UV filters in fish remains elusive, and the ecotoxicological risk for aquatic life is not known. Estrogenic activity *in vitro* has been shown for some UV filters in MCF-7 cells (Schlumpf *et al.*, 2001), recombinant cell lines (Mueller *et al.*, 2003; Schreurs *et al.*, 2002), and recombinant yeast systems carrying the human ERα (Kunz and Fent, unpublished; Routledge and Sumpter, 1997; Schultz *et al.*, 2000). Estrogenic activity has also been observed experimentally *in vivo* in rats (Durrer *et al.*, 2005; Schlumpf *et al.*, 2001; Seidlová-Wuttke *et al.*, 2004). In fish, high concentrations of 3-benzylidene camphor, 4-methyl-benzylidene camphor, and octyl-methoxycinnamate (Holbech *et al.*, 2002; Inui *et al.*, 2003) were found to be estrogenic after short-term exposure. Contrary to these studies, no estrogenicity was observed at 10 μM octyl-methoxycinnamate, benzophenone-3, homosalate, octyl dimethyl-*p*-aminobenzoic acid, butyl methoxydibenzoylmethane, and 1 μM 4-methyl-benzylidene camphor after short-term exposure of transgenic zebrafish (Schreurs *et al.*, 2002). Therefore the estrogenic activity of UV filters at low aqueous concentrations remains unclear.

The objectives of this study were to elucidate whether commonly used UV filters are estrogenic *in vitro* and *in vivo* in fish, to compare the *in vitro* activities in two *in vitro* systems carrying either a fish or the human ERα, and to compare the *in vitro* activity with the *in vivo* activity. We test the hypothesis that the estrogenic activity of chemicals is best assessed by the use of a tiered approach using a combination of *in vitro* and *in vivo* assays of the same phyla. As the rtERα has a different activity toward known estrogenic compounds than the hER (Le Guével and Pakdel, 2001; Pakdel *et al.*, 2000; Petit *et al.*, 1995), the question arises whether fish-based *in vitro* systems should be used for assessing estrogenicity in fish. Direct comparison of fish *in vitro* and *in vivo* activity demonstrates that the estrogenic activity *in vivo* may be partially predictable from the *in vitro* activity, although *in vitro* screening tends to overestimate the number of estrogenic compounds due to lack of or low metabolism. This indicates the need for a tiered approach, combining *in vitro* and *in vivo* assessments of hormonal activity of UV filters for ecological risk assessment.

EXPERIMENTAL SECTION

Chemicals

17β-Estradiol (E2) was purchased from Fluka AG (Buchs, Switzerland). Ultraviolet filters (Table 1) were obtained as follows. Benzophenone-1 (BP1), benzophenone-2 (BP2), benzophenone-3 (BP3), benzophenone-4 (BP4), 4′-hydroxybenzophenone (4HB), 4,4′-dihydroxybenzophenone (4DHB), 2,4,4′-trihydroxybenzophenone (THB), 4-aminobenzoic acid (PABA), benzylsalicylate (BS), phenlysalicylate (PS), octyl salicylate (OS), octocrylene (OC), and octyl dimethyl PABA (OD-PABA) were from Fluka AG; octylmethoxycinnamate (OMC), 3-(4′-methylbenzylidene-camphor) (4MBC), 3-benzylidene-camphor (3BC), and homosalate (HMS) were from Merck (Glattbrugg, Switzerland). Ethoxylated ethyl-4-aminobenzoate (PEG-25 PABA), a polymer consisting of ethyl 4-aminobenzoate and oxirane, was purchased from Induchem (Volketswil, Switzerland), and isopentyl-4-methoxycinnamate (IMC) was from Haarmann & Reimer (Holzminden, Germany). Bisimidazylate (BIM) was purchased form T. H. Geyer (Friedrichsthal, Germany). 4-tert-Butyl-4′-methoxydibenzoylmethane (BM-DBM) and 2-phenyl-5-benzimidazole-sulfonic-acid (PBS) were purchased from Aldrich (Fluka AG, Buchs Switzerland). 2,2-Methylenbis-phenol (ECL) was purchased from Ciba Speciality Chemicals (Basel, Switzerland), and Uvinul A plus B (UAB), a mixture of 35% 2-(4-diethylamino-2-hydroxybenzoyl)-benzoic acid hexylester and 65% OMC, was a gift from BASF AG (Wädenswil, Switzerland). All compounds were >99% pure. Stock solutions were made in ethanol and stored in the dark at 4°C. Analytical grade ethanol (EtOH, free of UV filters) was purchased from T. J. Baker (Stehelin AG, Basel, Switzerland). Bidestilled water was produced using a Jencons Autostill double D-ionstill destillator (Renggli AG, Rotkreuz, Switzerland).

Experiments *In Vitro* in Yeast

Recombinant yeast assay expressing the rainbow trout estrogen receptor alpha (rtERa assay). We investigated estrogenic activity of UV filters *in vitro* by applying a quantitiative β-galactosidase assay in liquid culture of recombinant yeast expressing the estrogen receptor of rainbow trout (rtERα) that was kindly provided by F. Pakdel, University of Rennes. We slightly modified the previously described assay procedure (Le Guével and Pakdel, 2001; Petit *et al.*, 1995). In general, the assay is based on transactivation of rtERα and induction of β-galactosidase leading to a color change. The estrogen-inducible expression system used is described in detail in (Le Guével and Pakdel, 2001). In brief, the yeast (*Saccharomyces cerevisiae*) genome carries a stably integrated DNA sequence of the rainbow trout estrogen receptor (rtERα). Yeast cells also contain expression plasmids carrying two estrogen-responsive elements (ERE) upstream of the yeast proximal iso-1-cytochrome c gene promoter fused to the lacZ gene (encoding the enzyme β-galactosidase). Thus, the induction is strictly dependent on the presence of rtERα and estrogens (Petit *et al.*, 1995). When an active ligand (*i.e.*, 17β-estradiol or an estrogenic UV filter) binds to the receptor, β-galactosidase is synthesized and secreted into the medium, leading to a catalytic hydrolysis of o-nitrophenyl-β-D-galactopyranosid (ONPG) and resulting in the development of a yellow color, which was measured as absorbance at 405 nm.

Preparation of rtERα assay media and yeast growth. The assay media were prepared as previously published (Le Guével and Pakdel, 2001; Petit *et al.*, 1995) and described (F. Pakdel, personal communication), with the following amendments: Complete Minimal Dropout Medium (CM) was prepared by adding 2% D-glucose instead of 1%. In addition to the CM medium, we used YPD growth medium (2% peptone enzymatic digest from meat, 2% D-glucose, and 1% yeast extract). Thus prior to the assay, yeast cell growth was calculated as described (Petit *et al.*, 1995), but with the

TABLE 1
Chemical Structures, Molecular Weight, and CAS Numbers of Compounds Analyzed

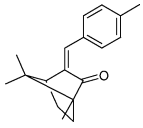
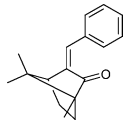
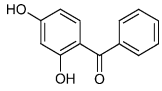
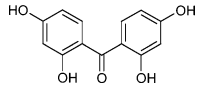
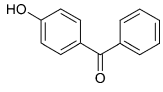
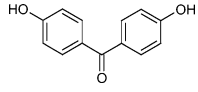
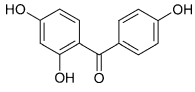
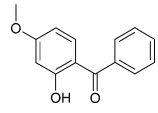
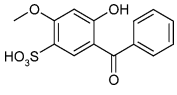
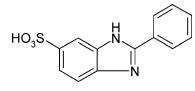
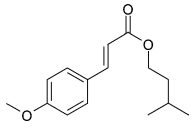
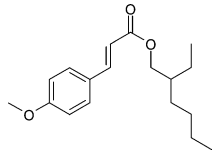
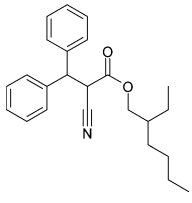
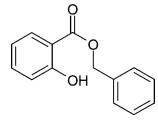
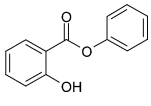
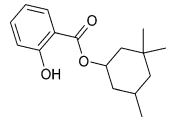
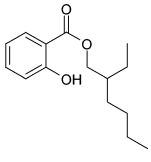
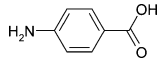
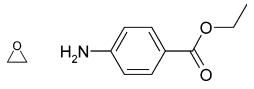
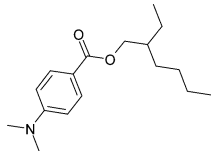
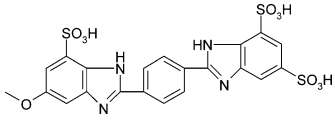
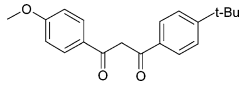
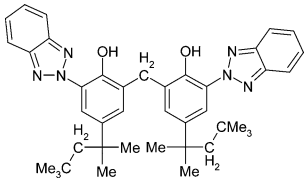
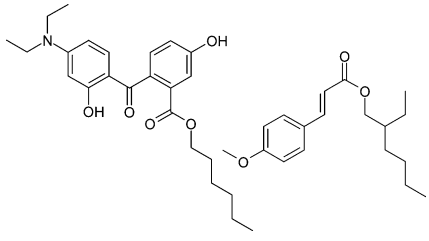
Compound MW, (CAS)	Chemical structure	Compound MW, (CAS)	Chemical structure
4MBC 254.37 (36861-47-9)		3BC 240.34 (15087-24-8)	
BP1 214.22 (131-56-6)		BP2 246.22 (131-55-5)	
4HB 198.22 (1137-42-4)		4DHB 214.22 (611-99-4)	
THB 230.22 (1470-79-7)		BP3 228.25 (131-57-7)	
BP4 308.31 (4065-45-6)		PBS 274.30 (27503-81-7)	
IMC 248.32 (71671-10-2)		OMC 290.40 (5466-77-3)	
OC 361.48 (6197-30-4)		BS 228.25 (118-58-1)	
PS 214.22 (118-55-8)		HMS 262.35 (118-56-9)	
OS 250.33 (118-60-5)		PABA 137.10 (150-13-0)	

TABLE 1—*Continued*

Compound MW, (CAS)	Chemical structure	Compound MW, (CAS)	Chemical structure
PEG25-PABA (113010-52-9) 44.05 (75-21-8) 165.2 (94-09-7)		OD-PABA 277.41 (21245-02-3)	
BIM 275.40 (180898-37-7)		BM-DBM 310.38 (70356-09-1)	
ECL 658.87 (103597-45-1)		UAB 397.52 (302776-68-7) 290.40 (5466-77-3)	

Abbreviations: MW, molecular weight; CAS, Chemical Abstracts Service; 4MBC, 3-(4′-methylbenzylidene-camphor); 3BC, 3-benzylidene-camphor; BP1, benzophenone-1; BP2, benzophenone-2; 4HB, 4-hydroxybenzophenone; 4DHB, 4,4′-dihydroxybenzophenone; THB, 2,4,4′-trihydroxybenzophenone; BP3, benzophenone-3; BP4, benzophenone-4; PBS, 2-phenly-5-benzimidazole-sulfonic-acid; IMC, isopentyl-4-methoxycinnamate; OMC, octyl-methoxycinnamate; OC, octocrylene; BS, benzylsalicylate; PS, phenlysalicylate; HMS, homosalate; OS, octyl salicylate; PABA, 4-aminobenzoic acid; PEG25-PABA, ethoxylated ethyl-4-aminobenzoate; OD-PABA, octyl dimethyl PABA; BIM, bisimidazylate; BM-DBM, 4-tert-Butyl-4′-methoxydibenzoylmethane; ECL, 2,2-methylenbis-phenol; UAB, Uvinul A plus B.

modification that yeast colonies from CM medium were inoculated in Erlenmeyer flasks containing 15 ml of YPD growth medium instead of CM medium, which lead to increasing growth rates and better assay performance.

rtERa assay procedure. The whole assay was performed as described in detail elsewhere (Le Guével and Pakdel, 2001; Petit *et al.*, 1995), but instead of hemolysis tubes in clear polystyrene, 96-well microplates (Greiner Bio-One, Huber AG, Basel, Switzerland) were used, leading to small modifications of the assay procedure according to Schultis and Metzger (2004). Thereby the centrifugation step after cell lysis was excluded and the lysed suspension, instead of the supernatant alone, was transferred to the flat-bottom 96-well plate. The protein measure (Petit *et al.* 1995) was replaced by quantifying yeast turbidity (A_{620}), to assess and correct for yeast growth and as a control for cytotoxicity. Cytotoxicity was manifested by significantly reduced yeast growth or even cell lysis, and it was determined by absorbance at 620 nm. High concentrations of some UV filters that lead to cytotoxicity were omitted from curve fitting and calculations.

For screening of the UV filters, a 96-well V-bottomed microtiter plate was filled with 100 µl/well *S. cerevisiae* cells in YPD culture. Three rows contained serially diluted positive control E2, one row the ethanol blank, and four rows the analyzed UV filter in quadruplicates with increasing concentrations, resulting in dose–response curves. After cell lyses the lysed suspension was transferred to a new flat-bottom 96-well plate (Greiner Bio-One, Huber AG, Basel, Switzerland), ONPG was added, and the estrogenic activity was measured as previously described (Le Guével and Pakdel, 2001; Petit *et al.*, 1995; Schultis and Metzger, 2004).

The hERα recombinant yeast was kindly provided by J. Sumpter, Brunel University, and the assay was performed according to Routledge and Sumpter (1996) and Kunz and Fent (unpublished). The yeast (*S. cerevisiae*) genome carries a stably integrated DNA sequence of the human estrogen receptor (hERα), and it also contains expression plasmids carrying EREs, regulating the expression of the reporter gene lacZ (encoding the enzyme β-galactosidase). Thus, when an active ligand (*i.e.*, E2 or an estrogenic UV filter) binds to the receptor, β-galactosidase is synthesized and secreted into the medium, leading to a color change of chromogenic substrate chlorophenol red β-D-galactopyranoside (CPRG) from yellow to red.

Experiments *In Vivo* in Fish

Fish. The 14-days fish experiments were conducted using juvenile, sexually undifferentiated fathead minnows (*Pimephales promelas*), between 2 and 3 months of age and with a total body length between 19 and 27 mm. This fish species has been chosen because of its frequent use in the field of endocrine disrupters and established techniques including vitellogenin (VTG) antibodies. The experimental procedure and duration was similar to that of Panter *et al.* (2002), who showed that estrogens and antiestrogens are detectable after 14 days of exposure by virtue of the VTG response.

Mixed-sex juvenile fathead minnows were received from the cultivator (Aquatic Research Organisms, Hampton NH, USA) and adapted for a minimum of 14 days in our laboratory in aquaria prior to the experiment. Fish were fed with Tetramin pellets (Tetra GmbH, Melle, Germany) twice a day with a quantity equivalent to 1% of body weight prior to the onset of experiments. During the experiments, fish were fed with brine shrimp (*Artemia salina*, Argent Chemical Labs, Redmond WA, USA) at a feeding rate of 1% of body weight twice a day.

Exposure. Fish were held in well-aerated reconstituted tap water medium (total hardness 160 mg/l as $CaCO_3$, total alkalinity 30 mg/l as $CaCO_3$,

conductivity 500 µs/cm) and a 16/8 h light/dark cycle at 25° ± 1°C. The studies were conducted using a 24-h static-renewal procedure with daily renewal of total aquaria water. For exposure, 10 randomly selected fish were each placed in stainless-steel tanks (10 liter) and exposed to individual UV filters for 14 days. Not all UV filters evaluated *in vitro* could be analyzed *in vivo*. To have a reasonable number of *in vivo* experiments, UV filters were selected as follows: either because they exhibited maximal estrogenic activities in our *in vitro* assays (BP1, BP2, 4DHB) or because they possessed submaximal (BP3, BP4, 3BC) or no (4MBC, OMC) estrogenic activity in our *in vitro* assays but were reported to be estrogenic by other studies, and because of their frequent use.

The first experiment was performed with 4MBC, 3BC, BP1, and BP2, and the second experiment was carried out with BP3, BP4, OMC, and 4DHB. In both experiments two controls, solvent control (SC, 1 ml ethanol in 10 liters of water) and positive control for estrogenic activity (100 ng/l E2), were included. Stock solutions of each chemical were prepared freshly in ethanol prior to the start of the experiment and added daily to the experimental water by mixing. The following nominal concentrations of UV filters were used: 10, 100, 500, 1000, and 5000 µg/l for BP1, BP3, BP4, OMC, and 4DHB, respectively; 10, 100, 500, and 1000 µg/l for 4MBC and 3BC, respectively; and 10, 100, 500, 1000, and 10,000 µg/l for BP2.

The concentrations were selected on the basis of environmental residues and included higher levels in order to span a large concentration range. Toxic side effects (*i.e.*, lethargy, uncoordinated swimming, loss of equilibrium, hyperventilation) were observed for fish exposed to 5000 µg/l BP3 and 1000 µg/l 4MBC, and the experiments were stopped at day 8 of exposure.

Physicochemical measurements and biological observations. Physicochemical parameters were determined daily. pH and oxygen saturation ranged between 7.2–7.9 and 6.5–8.3 mg/l, respectively, throughout the exposure period. Mortalities and abnormal behavior were recorded daily, and dead fish were removed from the tanks as soon as they were identified. On day 14 all fish were anesthesized with buffered tricaine methane sulfonate (MS-222, 100 mg/l with 200 mg $NaHCO_3$/l). Subsequently individual fish were measured, weighted, transferred into labeled Eppendorf tubes, frozen, and stored at −20°C for homogenization and VTG analysis.

Vitellogenin analysis. Fish were defrosted at 4°C and individually homogenized in ice-cold assay buffer (Biosense, Bergen, Norway) in a 1:2 ratio wet weight:buffer volume, using a Ultra Turax homogenizer (IKA, Huber + Co. AG, Reinach, Switzerland). The homogenates were centrifuged at 10,000 × *g* for 3 min at room temperature using a microcentrifuge (Eppendorf centrifuge 5415 D, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). The supernatant was withdrawn and immediately used for vitellogenin (VTG) analysis or frozen at −80°C until required for VTG analysis. Whole-body homogenates were assayed for VTG using a quantitative heterologous carp enzyme-linked immunosorbent assay, which has been shown to be highly reliable for VTG determination in the fathead minnow (Panter *et al.*, 2002; Tyler *et al.*, 1999). The commercially available quantitative carp vitellogenin ELISA kit, which is based on a sandwich ELISA format (Biosense), was used for determination of VTG in whole-body homogenates of individual fish and was conducted as described by Biosense. Purified carp VTG from blood plasma (Biosense) was used as a standard for quantitation according to the provider's description.

Analytical chemistry. For the duration of the experiment, four aliquots of 250 ml exposure waters were taken from the two highest and the two lowest concentrations of each UV filter and controls at the beginning (0 h) and prior to water renewal (24 h). The aliquots of the same concentration of UV filter were pooled for each UV filter at each concentration and time point in brown glass flasks, preserved by acidification using HCl to pH 2–3, and stored at 4°C until analysis. Chemical analyses of UV filter concentrations were carried out by high performance liquid chromatography (HPLC) and UV detection (Kunz *et al.* unpublished). Briefly, 25 or 250 ml of water samples, depending on sample concentration, were extracted and concentrated by solid phase extraction (SPE). The 2500× concentrated eluent was then analyzed by HPLC-DAD.

Data Processing and Statistical Analysis

Recombinant yeast assay. The absorbance measurement at 405 nm (ONPG) and 620 nm (turbidity) for the rtERα assay allowed for subsequent correction for turbidity (yeast growth) as follows:

$$\text{Corrected absorbance} = \text{chemical absorbance}_{405\,\text{nm}} - \text{chemical absorbance}_{620\,\text{nm}} - [\text{blank absorbance}_{620\,\text{nm}} - \text{blank absorbance}_{405\,\text{nm}}]$$

For all UV filters, the maximal response relative to the standard (=100%) were calculated. Thereby the height of the UV filter dose–response curve was expressed as a percentage of the maximal effect produced by the dose–response curve of E2.

High concentrations of some UV filters that inhibited growth of the yeast, or even lysed cells were omitted from curve fitting and calculations. For curve-fitting and EC50 calculations (GraphPad Software Inc., San Diego, CA, USA), the corrected absorbance values versus the logarithm of concentration were plotted, whereby the best fit from a number of nonlinear regression models was selected for final data analysis. In this study, we used the Hill equation (or sigmoidal dose–response with variable slope) to fit full dose–response curves, which reached the same height (≥80% maximal response) as the corresponding standard E2. Moderate (30–80% maximal response) and submaximal (<30% maximal response) dose–response curves were fitted using the best fit from a number of non-linear regression models. Coefficient of determination (R^2), residuals and 95% confidence intervals were calculated, and the runs test was carried out to verify that the fitted curve represents data correctly. Estrogenic potencies were calculated for all active UV filters. Thereby the EC50 of UV filters with full dose–response curves was divided by the EC50 of the E2 standard. For UV filters with submaximal dose–response curves, estrogenic potencies were estimated based on their EC50 values, despite differences in curve steepness and height when compared to the standards. In this way, a good approximation of the estrogenic potencies of submaximal UV filters was achieved.

Fish experiment. After testing the data distribution for normality by using the Kolmogorov-Smirnov test, means of wet weight and total length of individual fish were calculated, and data were analyzed by analysis of variance (ANOVA) followed by a Dunnett's Multiple Comparison test to compare the treatment means with respective controls. Means of VTG concentrations of individual fish were calculated, and data were analyzed with the non-parametric Kruskal-Wallis test, followed by a Dunn's Multiple Comparison test to compare the treatment means with respective controls. Statistical comparisons with the control were made using the SC as the overall control. The results are given as mean ± standard error of mean (SEM). Differences were considered significant at $p \leq 0.05$. All computations were performed with PRISM 4.0 (GraphPad Software Inc.).

RESULTS

Estrogenic Activity of UV Filters In Vitro

Ten of 23 analyzed UV filters and the UV filter metabolite 4HB were found to possess estrogenic activity in the recombinant yeast assay expressing the rainbow trout ERα. BP1, BP2, 4DHB, THB, 4HB, and PS were full rtERα agonists exhibiting full dose–response curves. They had maximal responses of 81–123% as compared to E2 (Fig. 1). Moderate, but clear dose–response curves were found for BS, BP3, and BP4, characterized by lower maximal responses of 43–74% (Fig. 1). Submaximal dose–response curves were observed with 3BC and OS with maximal responses of 27% and 15%, respectively

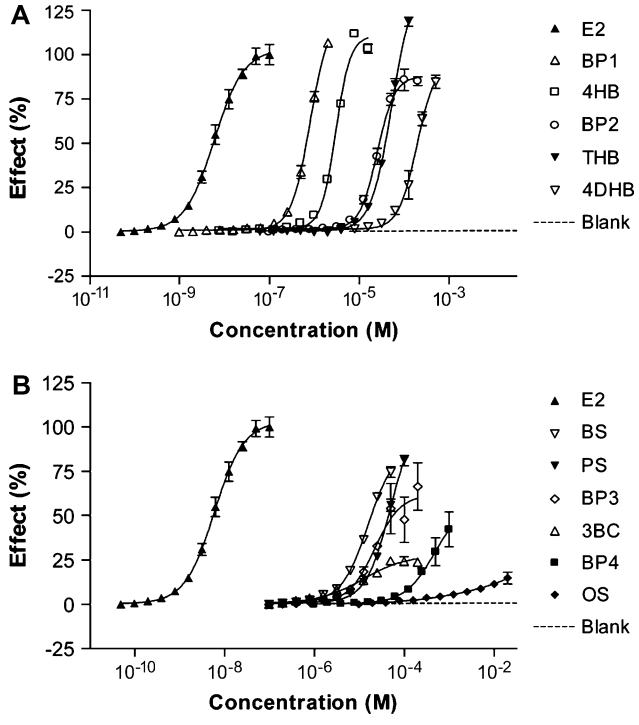


FIG. 1. Estrogenic activity of UV filters in the rtERα assay, shown in two panels (A, B) for clarity. Data shown are means ± SEM (three experiments with four replicates each). Effect (%) represents percentage of β-galactosidase induction of UV filters versus solvent (0%) and E2 (100%) controls. Results for inactive chemicals are not shown for clarity. Compound abbreviations see Table 1.

(Fig. 1). Table 2 shows the relative potencies of the UV filters compared to E2, as determined by their half-maximal induction activities (EC50). The most potent UV filter was BP1, which was only 87 times less potent then E2. Estrogenicity decreased in the following order 4HB > 3BC > BS > BP2 > BP3 > THB > PS > 4DHB > BP4 > OS. The estrogenic potencies were in the range of 390 to 24,750 times lower than E2. The remaining 12 UV filters, namely 4MBC, BIM, BM-DBM, ECL, HMS, IMC, OC, PABA, OD-PABA, PEG25-PABA, OMC, PBS, and UAB were inactive up to 2.5×10^{-2} M. In every assay we checked for potential cytotoxicity caused by the UV filter by routinely measuring yeast growth (620 nm) besides β-galactosidase activity (405 nm). Hence UV filter concentrations, which lead to reduced yeast cell growth or complete growth inhibition, were omitted from data analysis for hormonal activities. At high concentrations slight cytotoxicity occurred for BS ($\geq 5.04 \times 10^{-5}$ M), BP4 ($\geq 10.0 \times 10^{-4}$ M), 4DHB ($\geq 1.00 \times 10^{-3}$ M) and PS ($\geq 1.00 \times 10^{-4}$ M). Hormonal activity was therefore assessed at non-cytotoxic concentrations only.

Comparison with hERα. The same UV filters found active in our present study with recombinant yeast expressing the rainbow trout ERα were previously found active in a recombinant yeast system expressing the human estrogen receptor alpha (hERα). With this system, we investigated 17 UV filters and one metabolite for their multiple hormonal activities such as estrogenicity, antiestrogenicity, androgenicity, and antiandrogenicity *in vitro* (Kunz and Fent, unpublished). The only exception was OS that exhibited minimal estrogenic activity in the rtERα only. The activities of BP1, 3BC, and the salicylates were relatively higher with rtERα than with hERα, but they were lower for the remaining benzophenones, displaying lower EC50 values with hERα. BP1 and 4HB showed strongest activities in both receptor systems. The rankings of the other UV filters differed between the receptor systems, however. In the hERα assay benzophenone derivatives were the most potent compounds. Benzophenone-, camphor-, and salicylate derivatives were most potent in the rtERα assay. The maximal responses of estrogenic compounds in the rtERα assay were in most cases higher than in the hERα, with only BP2 and 4DHB as exceptions (Table 2). A direct comparison of the two assays is shown in Figure 2. BP1 as the most potent UV filter in both assays displayed an estrogenic activity only 87 times less than that of E2 with rtERα and 5000 times less with hERα. In particular, the relative activity of 3BC was higher in the rtERα assay. In contrast to the relatively higher activity of UV filters in the rtERα assay, the hERα assay was 62 times more sensitive toward E2.

Estrogenic Activity of UV Filters in Fish In Vivo

Measured exposure concentrations. To determine actual effect concentrations and to get an estimate of concentration decrease, concentrations of UV filters in aquaria waters were measured at the beginning of exposures (0 h) and 24 h later, prior to water renewal at the lowest and the two highest exposure concentrations. Concentrations decreased during exposure, but to a variable extent for different compounds. Table 3 shows that actual concentrations determined by HPLC analysis were close to nominal. After 24 h before water renewal, concentrations decreased to various degrees (0–32%) depending on compound and concentration. The different concentration decreases are a result of different physicochemical properties of UV filters (lipophilicity) and uptake by fish.

Effects of UV filters on fish survival, weight, and length. No mortality was observed in control, solvent control (SC), and positive control (E2) exposed fish in either experiment. The UV filters did not affect survival during exposure, except at the highest concentrations of 4MBC, 3BC, and BP1. After 8 days of exposure, two fish died at 753 µg/l 4MBC (survival 80%), and the experiment was stopped. At 953 µg/l 3BC and 4919 µg/l BP1, one fish each was found dead at day 12 and day 11, respectively. Fish at 8783 µg/l BP2 showed some signs of edema at the end of exposure.

During the 14-day exposures, all control, SC, and E2 fish grew as determined by increase in wet weight and total body length (Table 4). At low concentrations of UV filters no

TABLE 2
Comparison of *In Vitro* (rtERa, hERa) and *In Vivo* Effect Concentration

	In vivo		*In vitro*								
	VTG Induction		rtER assay EC50				hER assay EC50				Sensitivity Ratio EC50 (rtER/hER)
	(μg/l)	(M)	(μg/l)	(M)	Maximal response	Potency (1/...)	(μg/L)	(M)	Maximal response	Potency (1/...)	
E2	0.1	3.67×10^{-10}	4.93	$1.81 \times 10^{-8} \pm 5.12 \times 10^{-9}$	100%	1	0.08	$2.91 \times 10^{-10} \pm 1.19 \times 10^{-10}$	100%	1	62.00
4MBC	*n.e.*		*n.e.*				*n.e.*				—
3BC	953	3.96×10^{-6}	2927.00	1.22×10^{-5}	27%	960	74,443.00	3.10×10^{-4}	21%	1.3×10^{6}	0.04
	435	1.81×10^{-6}									
BP1	4919	2.30×10^{-5}	171.26	7.99×10^{-7}	114%	87	247.15	1.15×10^{-6}	96%	5000	0.70
BP2	8783	3.57×10^{-5}	6141.00	2.49×10^{-5}	88%	2690	2684.00	1.09×10^{-5}	91%	21,000	2.30
4DHB	*n.e.*		36,867	1.72×10^{-4}	88%	23,340	15,727.00	7.34×10^{-5}	91%	170,000	2.30
4HB	—		586.73	2.96×10^{-6}	111%	390	360.56	1.82×10^{-6}	108%	16,000	1.60
THB	—		10,506.00	5.30×10^{-5}	123%	7890	1818.00	9.17×10^{-6}	103%	27,730	5.80
BP3	*n.e.*		4999	2.19×10^{-5}	62%	3470	4237.00	1.86×10^{-5}	18%	45,000	1.20
BP4	*n.e.*		91,846.00	2.98×10^{-4}	43%	24,750	29,241.00	9.48×10^{-5}	6%	380,000	3.10
IMC	—		*n.e.*				*n.e.*				—
OMC	*n.e.*		*n.e.*				*n.e.*				—
OC	—		*n.e.*				*n.e.*				—
BS	—		2614.00	1.15×10^{-5}	74%	1800	37,872.00	1.66×10^{-4}	12%	860,000	0.07
PS	—		6704.00	3.13×10^{-5}	81%	8200	23,648.00	1.10×10^{-4}	32%	480,000	0.30
HMS	—		*n.e.*				*n.e.*				—
OS	—		964,772.00	3.85×10^{-5}	15%	570,000	*n.e.*				—
PABA	—		*n.e.*				*n.e.*				—
PEG25 PABA	—		*n.e.*				*n.e.*				—
OD-PABA	—		*n.e.*				*n.e.*				—
BIM	—		*n.e.*				*n.e.*				—
BM-DBM	—		*n.e.*				*n.e.*				—
ECL	—		*n.e.*				*n.e.*				—
UAB	—		*n.e.*				*n.e.*				—

Abbreviations: E2, 17β-estradiol; EC50, the concentration of the compound exhibiting 50% of its total effect. n.e., no effect. Values for E2 standard are given in mean S.E.M. of 10 (rtERα) or 9 (hERα, Kunz *et al*., unpublished) experiments, respectively with three replicates each. EC50 values of compounds are from three experiments with four replicates each. Potency = EC50 compound/EC50 E2 is calculated from mean values of each single experiment; the used EC50 values for E2 in each experiment are not listed here for reasons of simplicity. Chemical concentrations shown for the *in vivo* assay are those at which VTG was significantly induced relative to the solvent control. For compound abbreviations see Table 1.

significant differences from controls were observed. Length gain was significantly decreased for 435 and 953 μg/l 3BC and 4MBC (Table 4), however. No difference occurred in wet weight and mean length in the SC and E2. 3BC and 4MBC led to a dose-related decrease in the weight gain and body length at 435 and 953 μg/l, and 415 and 753 μg/l, respectively. No decreases in body weight gain and body length were observed with all other UV filters at all exposure concentrations, except for 4919 μg/l BP1 after 14 days of exposure.

Estrogenicity of UV filters. Significant VTG induction occurred in fish exposed to ng/l E2. Mean whole-body VTG content was 2600 μg/ml and was highly induced compared to the water and solvent control having a residual level of 0.3 μg/ml. Dose-dependent increases in VTG were observed in fish exposed to 3BC, BP1, and BP2 (Fig. 3). 3BC showed higher VTG induction and at lower concentration compared to BP1 and BP2. Dose-related significant VTG induction occurred at 3BC concentrations of 435 μg/l (407 μg VTG/ml) and 953 μg/l (1753 μg VTG/ml). Concentration-related VTG induction was also found after exposures to higher concentrations of BP1 and BP2. Although increased at medium concentrations, VTG induction was significant only at the highest concentrations of BP1 and BP2, namely at 4919 μg/l BP1 (907 μg VTG/ml) and 8783 μg/l BP2 (1504 μg VTG/ml). The UV filters BP3, BP4, and 4DHB did not result in a significant VTG induction at all exposure concentrations in fish, although they showed submaximal estrogenic activity *in vitro*. 4MBC and OMC, which showed no estrogenicity *in vitro*, were not estrogenic *in vivo*. Therefore, three of five UV filters that exhibited estrogenic activity *in vitro* were also estrogenic *in vivo*.

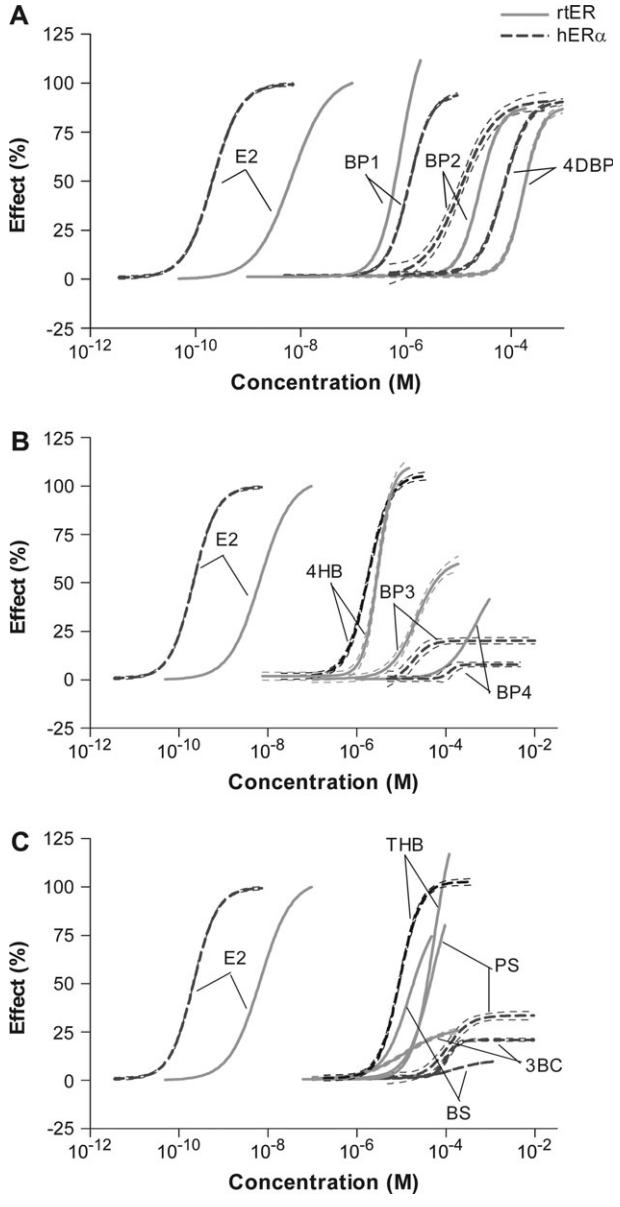


FIG. 2. Comparison of estrogenic activity of UV filters between the rtERα (bold lines) and the hERα (dashed lines) assay, shown in three panels (A, B, C) for clarity. Data shown are means and 95% confidence intervals (three experiments with four replicates each). Effect (%) represents percentage of β-galactosidase induction of UV filters versus solvent (0%) and E2 (100%) controls. Results for inactive chemicals are not shown for clarity. Compound abbreviations see Table 1.

TABLE 3
Nominal and Measured Water Concentrations of Analyzed UV Filters

	Exposure concentrations				
	Nominal	Measured			
	(μg/l)	0 h (μg/l)	24 h (μg/l)	Median (μg/l)[a]	After 24 h (%)[b]
3BC	10	9.5 ± 0.3	8.0 ± 1.2	9	84
	500	516.7 ± 36.7	352.5 ± 130.8	435	68
	1000	1070.0 ± 28.3	835.0 ± 49.5	953	78
BP1	10	9.8 ± 0.1	8.1 ± 0.2	9	82
	1000	1032.4 ± 54.8	930.0 ± 14.1	981	90
	5000	5191.7 ± 58.7	4647.1 ± 221.4	4919	90
BP2	10	10.7 ± 0.6	9.9 ± 0.9	10	93
	1000	1102.5 ± 9.8	1031.7 ± 17.0	1067	94
	10,000	9747.3 ± 557.9	7818.5 ± 104.5	8782	80
4MBC	10	9.6 ± 1.8	7.4 ± 0.4	9	77
	500	492.4 ± 102.7	337.5 ± 17.7	415	69
	1000	826.1 ± 189.4	680.0 ± 198.0	753	82
OMC	10	8.8 ± 0.1	6.5 ± 0.7	8	74
	1000	1012.5 ± 165.6	765.0 ± 63.6	889	76
	5000	5450.0 ± 282.8	4600.0 ± 141.4	5025	84
BP3	10	13.5 ± 1.1	9.7 ± 0.2	12	71
	1000	879.5 ± 115.0	652.2 ± 65.4	766	74
	5000	4175.0 ± 247.5	3625.0 ± 106.1	3900	87
BP4	10	11.5 ± 0.9	10.8 ± 0.8	11	94
	1000	1068.2 ± 64.4	1027.7 ± 99.2	1048	96
	5000	5158.9 ± 425.2	4634.3 ± 26.4	4897	90
4DHB	10	11.8 ± 1.0	9.0 ± 0.1	10	76
	1000	901.9 ± 48.4	899.0 ± 42.9	900	100
	5000	5388.5 ± 2.7	4633.0 ± 232.4	5011	86

[a]Median of actual concentrations at 0 h and 24 h.
[b]Percentage of actual concentration at 24 h relative to 0 h.

DISCUSSION

In the present study we show that it is most appropriate to determine the endocrine-disrupting activity of chemicals both *in vitro* and *in vivo*, preferably in a tiered approach, as no single assay may be best suited to determine the hormonal activity of a compound and because of species differences. In this way, we demonstrate for the first time that as many as 10 of 23 commonly used UV filters are estrogenic in an *in vitro* yeast assay carrying a fish ER (rtER). Compared to our results in the recombinant yeast carrying the hERα, where we investigated 17 UV filters and one metabolite for estrogenicity, antiestrogenicity, androgenicity, and antiandrogenicity *in vitro* (Kunz and Fent, unpublished), we found that all compounds, except OS, were equally estrogenic in both assays, despite lower activity of E2 in the rtERα assay. In fish we demonstrated that three of eight UV filters were estrogenic *in vivo*. Comparing *in vitro* activities in two systems with fish *in vivo* activity, we found the rtERα *in vitro* data more accurate than the hERα data for prediction of the *in vivo* activity. Hence estrogenic activity of chemicals is best assessed by the use of a tiered approach with a combination of *in vitro* and *in vivo* assays of the same species.

In Vitro *Activity in the rtERα Assay*

We found 10 of 23 compounds exhibiting estrogenic activities in the rtERα assay with different maximal responses

TABLE 4
Body Weight and Length of Exposed Fish after 0 and 14 Days of Exposure

	Exposure (μg/l)	Body weight (mg)	Body length (mm)		Exposure (μg/l)	Body weight (mg)	Body length (mm)
Controls	Day 0	62.0 ± 24.4	18.9 ± 2.2	Controls	Day 0	161.3 ± 45.7	27.4 ± 2.1
	Water	211.3 ± 91.1	29.4 ± 2.8		Water	313.1 ± 80.0	33.8 ± 3.1
	Solvent	238.0 ± 88.8	30.8 ± 3.5		Solvent	277.0 ± 62.2	32.0 ± 2.2
	E2	249.0 ± 111.8	29.6 ± 4.1		E2	282.9 ± 70.7	32.4 ± 2.6
4MBC	9	272.4 ± 82.9	31.1 ± 2.9	BP3	12	313.9 ± 59.9	34.6 ± 2.0
	100	237.3 ± 60.6	30.4 ± 2.4		100	317.5 ± 105.4	33.3 ± 3.7
	415	103.9 ± 40.8**	23.6 ± 2.6**		500	281.6 ± 123.7	32.7 ± 3.5
	753	43.1 ± 25.2**	17.1 ± 3.6**		766	257.3 ± 51.1	32.0 ± 1.7
					3′900	134.6 ± 36.6**	27.9 ± 2.2*
3BC	9	335.9 ± 128.9*	32.8 ± 3.1				
	100	258.9 ± 95.9	30.9 ± 3.4	BP4	11	330.5 ± 114.6	34.2 ± 4.4
	435	115.1 ± 37.4**	24.7 ± 2.4**		100	326.4 ± 102.2	33.4 ± 3.0
	953	95.8 ± 25.8**	23.7 ± 1.8**		500	309.2 ± 69.3	34.1 ± 1.9
					1′048	299.9 ± 57.8	33.5 ± 1.7
BP1	9	246.5 ± 77.0	30.0 ± 2.6		4′897	375.4 ± 97.5*	35.5 ± 2.8*
	100	273.1 ± 141.2	31.7 ± 4.6				
	500	240.4 ± 67.8	30.5 ± 2.1	OMC	8	303.3 ± 114.5	33.4 ± 3.6
	981	245.0 ± 90.0	29.4 ± 3.8		100	266.7 ± 54.5	32.4 ± 2.5
	4′919	121.9 ± 46.9*	24.6 ± 3.2**		500	283.3 ± 62.6	32.8 ± 1.8
					889	303.2 ± 65.8	33.2 ± 2.2
BP2	10	272.9 ± 125.7	30.9 ± 4.9		5′025	267.4 ± 60.5	32.4 ± 2.1
	100	264.5 ± 116.9	30.6 ± 4.1				
	500	247.6 ± 73.7	30.5 ± 2.2	4DHB	10	321.8 ± 70.2	34.7 ± 1.1
	1′067	293.4 ± 113.4	32.1 ± 3.6		100	316.9 ± 111.4	34.0 ± 3.4
	8′783	171.4 ± 35.0	25.9 ± 1.8**		500	340.9 ± 102.2	34.4 ± 3.0
					900	351.4 ± 93.7	34.5 ± 2.6
					5′011	371.5 ± 97.0	35.5 ± 3.0*

E2: 17β-estradiol.
*Significantly different from solvent control at $p < 0.05$.
**Significantly different from solvent control at $p < 0.01$.

and dose–response curves. The range of moderate to full dose–response curves can be explained by the molecular structures. Ultraviolet filters displaying full dose–response curves are characterized by at least one ring-substituted hydroxyl group. They display lower maximal responses with increasing molecular symmetry. Additional substituents on the phenolic ring have a diminishing effect on the maximal estrogenic responses in both ERα systems (Kunz and Fent, unpublished; Routledge and Sumpter, 1997). This is the case for BP3, BP4, BS, OS, and 3BC, which are substituted with methoxy-, sulfonic acid-, benzyl, octyl, or camphor groups, possibly indicating only partial agonism. Like those compounds with very large molecular structures that prevent uptake into cells, inactive UV filters had, with very few exceptions, only one non-hydroxylated ring that was connected and/or attached to other substituents such as ethoxy, alkyl-, amino-, cyano-, or methoxy-groups, which were shown to significantly decrease the chemical's affinity for the rtERα, as previously shown for hERα (Blair *et al.*, 2000). The structural basis of the estrogenic UV filters found in our study is in line with recent findings on structure–activity relationships of structurally similar chemicals in hER-systems (Miller *et al.*, 2001; Routledge and Sumpter, 1997; Schultz *et al.*, 2000).

Comparison Between Rainbow Trout and Human ERα

We were interested in elucidating whether the structural differences between human and rainbow trout ERα (Petit *et al.*, 1995, 2000) were responsible for functional differences. Homologies in amino acid sequences between hERα and rtERα are variable, depending on domain (Pakdel *et al.*, 1990). The most highly conserved region is the C domain (92% homology), which is responsible for DNA binding and dimerization (Petit *et al.*, 2000). Whereas rtERα and hERα have similar binding affinities to an estrogen response element, the rtERα C domain is responsible for a weaker DNA binding stability. The E domain shares 60% similarity with rtERα and hERα and contains the hormone-binding domain. Petit *et al.* (1995) found that the rtERα has a lower affinity for E2 than the hERα. This was further demonstrated for 17β-estradiol, estrone, and 17β-ethinylestradiol (Le Guével and Pakdel, 2001) and is confirmed by the present study; the E2

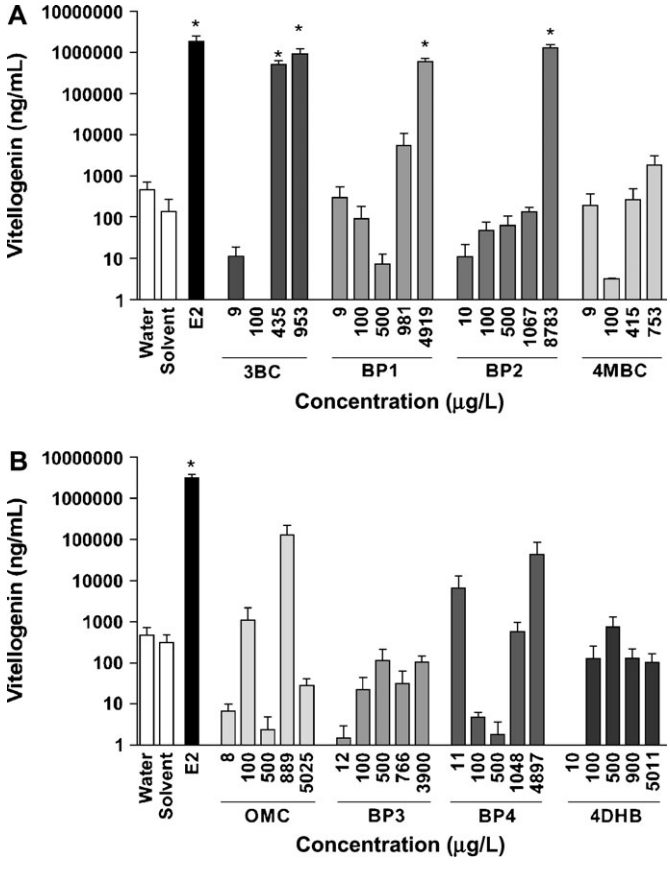


FIG. 3. Vitellogenin concentration in juvenile fathead minnows exposed to eight UV filters. Values are means ± SEM ($n = 10$). Asteric denotes a significant difference from control (solvent) at $p \leq 0.05$. Concentrations given as actual median measured, except 100 and 500 µg/l.

concentration necessary to induce 50% activity was 62 times higher in the rtERα assay. The weaker magnitude of E2 stimulation mediated by rtERα is attributed to the lower DNA-binding stability and not to structural differences between the two ER (Petit *et al.*, 2000).

As for most of the UV filters, relative sensitivities of rtERα and hERα systems varied very little (one order of magnitude), indicating that the main difference between the two receptors is their sensitivity for E2 (Table 2). Differences between the two ER occurred for the salicylate derivatives (PS, BS, OS), which showed several times higher activity in rtERα, and for 3BC, which showed as much as 1300 times higher activity than in the hERα. Maximal responses were generally higher in the rtERα assay, except for BP2 and 4DHB. This indicates a higher relative sensitivity and weaker partial agonism in the rtERα of compounds showing submaximal activity.

Thus, in contrast to the lower activity of E2, the activity of some UV filters is relatively higher in the rtERα assay. This cannot be fully explained by a lower DNA-binding stability found by Petit *et al.* (2000) for estradiol, but is rather attributed to structural differences of the two ER and the molecular structure of the UV filters interacting with the ER. This points toward a slightly different substrate binding specificity of the fish and human ERα based on differences in the binding domain of the two receptors. In addition, differences in the transactivation process such as dimerization and DNA-binding capacity may also account in part for the different relative sensitivities. Forthcoming studies focusing on UV filter receptor binding and influence on the transactivation process will elucidate the reasons for the differing sensitivities of the fish and hERα.

Comparison with Other In Vitro *Studies*

The estrogenicity of UV filters found in our study with rtERα is consistent with results obtained *in vitro* in human ER systems, although relative sensitivities may differ. Estrogenicity of some salicylate and camphor derivatives have been reported in mammalian systems such as recombinant yeast (Kunz and Fent, unpublished; Miller *et al.*, 2001; Mueller *et al.*, 2003), receptor binding assays (Blair *et al.* 2000; Mueller *et al.*, 2003; Schlumpf *et al.*, 2004), proliferation of MCF-7 cells (Schlumpf *et al.*, 2001, 2004), and reporter gene induction in transfected cell lines (Schreurs *et al.*, 2002; Suzuki *et al.*, 2005; Yamasaki *et al.*, 2003). The estrogenicity of BP1, BP2, BP3, and 3BC found in our rtERα assay is consistent with findings in MCF-7 cells (Schlumpf *et al.*, 2001, 2004), reporter hERα/HeLa cells (Yamasaki *et al.*, 2003), MCF7 reporter cells (Suzuki *et al.*, 2005), and HEK293 cells (Schreurs *et al.*, 2005). Our results are also consistent with the hERα cell assay for BP3, but not for 4MBC in the HEK 293 reporter gene assay. In addition, 3BC, HMS, and 4MBC showed activity in the hERα assay (Schreurs *et al.*, 2002).

In fish, at least two ER subtypes, ERα and ERβ, occur, and in zebrafish, a third form has been reported (Menuet *et al.*, 2002). At present it is not known to what extent UV filters interact with these receptors. Reasons for differences between results obtained in our study with the rtERα assay and other *in vitro* assays are, first, that UV filters may be active toward the ERβ, but not the ERα. In the MCF-7 and other human cells, active UV filters may interfere with both hERs. 4MBC was estrogenic in the MCF-7 cells (Schlumpf *et al.*, 2001), but it did not exhibit estrogenic activity toward the rtERα in the present study, a finding similar to that previously reported from our experiments with the hERα (Kunz and Fent, unpublished), based on the fact that 4MBC binds preferably to the ERβ (Schlumpf *et al.*, 2004). This is also the case for HMS (Schreurs *et al.*, 2002). In addition, yeast has only a low capability for metabolism; therefore metabolites of UV filters binding to the ER are not identified by the rtERα assay. The differences may also depend in part on different binding activities of the ERs of different species and on differences between *in vitro* assays and their varying capabilities to activate a chemical metabolically. This leads to the conclusion that species differences in the estrogenic activity occur and that one

in vitro assay alone is not sufficient for assessing the estrogenicity of chemicals and fully characterizing their estrogenic potential. Moreover homologous *in vitro* systems are more reliable for predicting *in vivo* activity.

Comparison of In Vivo *Activities*

Our *in vivo* experiments demonstrate that of eight analyzed UV filters, three—3BC, BP1, and BP2—showed estrogenic activity in fathead minnows. 3BC led to dose-dependent induction of VTG at lower concentrations (435, 953 μg/l) than BP1 (4919 μg/l) and BP2 (8783 μg/l). 4MBC, OMC, BP3, BP4, and 4DHB did not induce VTG up to the highest concentrations in the range between 753 μg/l (4MBC) and 5010 μg/l (4DHB). Schreurs *et al.* (2002) observed no estrogenicity in transgenic zebrafish exposed for 96 h at 10 μM of OMC (2.90 mg/l), OD-PABA (2.77 mg/l), HMS (2.62 mg/l), BP3 (2.28 mg/l), and 1 μM of 4MBC (0.25 mg/l), which is consistent with our data with these UV filters analyzed at similar concentrations but for a longer period of time. Injection of high concentrations of 3BC (27, 68, 137 mg/kg and higher) induced VTG in rainbow trout (Holbech *et al.*, 2002). In medaka, estrogenic activity of 4MBC and OMC was observed only at about 200 times higher concentrations (Inui *et al.*, 2003), but it was not found at 20 times higher concentrations than in our study. This may be related to species-specific differences in VTG induction. The relative degree of VTG induction is species-specific, as shown for rainbow trout that reacted with higher VTG induction to endocrine disrupters than roach (Routledge *et al.*, 1998).

The UV filters 4MBC, BP3, 4DHB, and OMC exhibited estrogenicity *in vivo* in rats (Mueller *et al.*, 2003; Schlumpf *et al.*, 2001, 2004; Yamasaki *et al.*, 2003), but this was not observed in our fish study. The differences can be explained by species differences in metabolism and different affinities to the ERs, as 4MBC and HMS preferably bind to the ERβ (Schlumpf *et al.*, 2004; Schreurs *et al.*, 2002). Whether this is the case in fish is not known. Most likely, the differences are based on the different metabolic capabilities of fish compared to rats, but also on lower exposure concentrations. In our fish experiments, UV filter levels in water were lower than in rats exposed to UV filters via feed.

Comparison of In Vitro *and* In Vivo *Activity*

The estrogenic activity *in vitro* was matched *in vivo* for most UV filters. 4MBC and OMC exhibited neither estrogenic activity in *in vitro* transactivation assays carrying either the hERα or rtERα nor in fish *in vivo*. 3BC, BP1, and BP2 showing activity *in vitro* were also active *in vivo*. Both *in vitro* and *in vivo*, they possessed the highest potencies of the tested UV filters. The *in vitro* activity of BP1 (EC50 7.9×10^{-7} M) was higher than that of 3BC (1.2×10^{-5} M), whereas BP2 was the least active of these three compounds, both *in vitro* and *in vivo* (Figs. 1–3). The *in vivo* activity of 3BC was higher than expected from its *in vitro* potency in the rtERα assay, where it was the second most potent UV filter after BP1. Being only 87 times less active than E2 in the rtERα assay, BP1 showed only weak *in vivo* activity in fathead minnows. This might be explained by its higher metabolism and lower lipophilicity (and lower bioaccumulation potential) compared to the more lipophilic 3BC. Furthermore, the relatively higher estrogenicity of 3BC *in vivo* might be based on the higher binding activity of 3BC to the ERβ than to ERα of fathead minnows, as 3BC binds preferentially to human recombinant ERβ, and only slightly to ERα (Schlumpf *et al.*, 2004). There are no indications that metabolites of 3BC are more active than the parent compound. On the basis of the rtERα assay, the relatively low *in vivo* activity of BP2 is consistent with our *in vitro* data. This might also be the reason why 4DHB, BP3, and BP4 possess lower rtERα potencies. The estrogenic activity of most benzophenones and salicylates seems to be abolished *in vivo* because of metabolism.

In comparisons of the potency rankings of UV filters for the rtERα and the hERα assay, the data clearly demonstrate that the rtERα *in vitro* data are more accurate than the hERα data in predicting the *in vivo* activity. This indicates that hormonal activity of UV filters should be assessed by a suite of species-related *in vitro* and *in vivo* assays in which the *in vitro* assay should be able to predict to most potent compounds for further *in vivo* testing. Differences in *in vitro* and *in vivo* activities, which we nevertheless observed in our fish-based assays, are attributable to metabolism, and also to different activities to different ERs in fish. Our approach using rtERα *in vitro* and fathead minnow *in vivo* may cover species differences in fish. Perhaps using the same fish species (rainbow trout) in the *in vivo* assay as in the *in vitro* assay would have resulted in more comparable results between the *in vitro* and *in vivo* assays.

Environmental Consequences

In the environment only a few UV filters such as OC, 4MBC, BP3, and BM-DBM have been analyzed to date. In lake water, BP3, 4MBC, and OC occurred at concentrations of 80–125, 60–80, and 22–27 ng/l, respectively, in the upper layer of a bathing lake (Poiger *et al.* 2004), but they were lower in other lakes (Balmer *et al.*, 2005). Concentrations in treated wastewater were 0.06–2.7 (4MBC), 0.01–0.7 (BP3), 0.01–0.1 (OMC), and 0.01–0.27 μg/l (OC) (Balmer *et al.*, 2005). Residues of 4MBC, OMC, BP3, and HMS were also found in muscle tissue of fish from a German lake at levels between 21 and 3100 ng/g lipid (sum of all UV filters 2 μg/g in perch and 0.5 μg/g in roach), and between 25 and 166 ng/g lipid in 10 whitefish from Swiss lakes (Balmer *et al.*, 2005). However, 3BC, BP1, and BP2, which were found in our study to be estrogenic, have not yet been analyzed in aquatic systems. If they were in the same range, VTG induction after short-term exposure to a single UV filter would probably not pose a hazard to fish. However, different UV filters may act additively

(Heneweer *et al.*, 2005), as indicated for other endocrine disrupters (Routledge *et al.*, 1998). Moreover, long-term exposure to UV filters may affect fish reproduction at much lower concentrations.

As it is not known to what extend these UV filters occur in the environment and in fish, comprehensive hazard and risk assessment is premature. Forthcoming studies should determine environmental concentrations of estrogenic UV filters and to relate them to effect concentrations. For hazard and risk assessment, potential effects on reproduction, fecundity, and fertility in fish are necessary, as are bioaccumulation studies. Moreover, UV filters may have multiple hormonal activities such as antiestrogenicity, androgenicity, and antiandrogenicity, in addition to estrogenicity (Kunz and Fent, unpublished). Whether these multiple hormonal activities are reflected *in vivo* in fish, and whether reproduction effects occur, is now under investigation in our laboratory.

CONCLUSIONS

Considering the vast number of compounds to be tested for possible endocrine activity, it is important to employ appropriate *in vitro* systems. They are cost effective and allow for rapid screening of a large number of compounds, but they have limitations that may result in unreliable predictions. In the present study we show that it is most appropriate to determine the endocrine-disrupting activity of chemicals both *in vitro* and *in vivo*, as no single assay appears to be best suited to determine the hormonal activity of a compound, and because there are species differences. We propose that receptor-based assays with related or even the same species should be used for *in vitro* screening prior to *in vivo* testing. In this tiered approach, the predictive power of *in vitro* systems is enhanced, and cost intensive *in vivo* studies can be reduced by employing species-specific *in vitro* assays. This leads to the conclusion that an environmental risk assessment should be based on combined, complementary, and appropriate species-related *in vitro* and *in vivo* assays for hormonal activity.

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